Chapter 6

EXOPOLYSACCHARIDES OF rRNA GROUP I PSEUDOMONADS

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ABSTRACT

Bacterial and algal polysaccharides are used for a variety of applications both in food and nonfood industries. Alginate, a glycuronan isolated from brown algae, is used for a variety of commercial applications. Bacterial alginates, present as exopolysaccharides (EPS's), may be viable alternatives to algal alginates for certain of these uses. During the past several years we have examined over 200 strains of plant-associated bacteria belonging to rRNA group I of the genus *Pseudomonas* for the ability to produce EPS's including alginate. Alginate production was demonstrated for several *Pseudomonas* species. In addition, three novel acidic EPS's were identified and their structures determined. Levan (a polyfructan) was also produced by many of the strains when sucrose was the primary carbon source. Based on Southern blotting and hybridization techniques using probes for biosynthetic alginate genes, a similar pathway to alginate synthesis appears to be used by the plant-associated pseudomonads and human pathogenic *P. aeruginosa*. Alginate production was enhanced by addition of NaCl, sorbitol or ethanol to culture media.

INTRODUCTION

Under natural conditions, bacteria are surrounded by a glycocalyx which is primarily composed of exopolysaccharide (EPS) (1). Bacterial EPS's are usually heteroglycans with small oligosaccharide repeating units. With some exceptions, bacterial EPS's are anionic and of high molecular mass. The acidic nature of EPS is

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usually due to the presence of uronic acids, phosphoric diester groups or pyruvate acetals (2).

Three bacterial EPS's (xanthan gum, dextran, and gellan gum) are commercially available. Xanthan gum, produced by the plant pathogen *Xanthomonas campestris*, is the major microbial polysaccharide for industrial use. It was put into commercial production in 1964 and has a wide variety of food and nonfood industrial applications due to its unique rheological properties (3). Dextran, for industrial use, is produced primarily by *Leuconostoc mesenteroides*. Dextran and its various derivatives have found their greatest applications in the pharmaceutical and fine-chemical industries (3). The most recent entry into the bacterial EPS market is gellan gum (4). Gellan gum is produced by *Auromonas elodea* (formerly *Pseudomonas elodea*). It can be used as an agar substitute for nutrient media, with the advantage of giving a clear gel, and also was recently approved for some food uses.

Until recently very little was known about the nature of EPS's produced by bacteria of the genus *Pseudomonas*. The genus *Pseudomonas* is divided into five rRNA groups based on rRNA-DNA homology studies (5). We have concentrated most of our efforts on rRNA homology group I. The majority of group I species produce fluorescent pigments when grown under iron-limiting conditions, and thus are called fluorescent pseudomonads. Group I species include plant pathogens, human and animal pathogens and saprophytes. One species (*P. aeruginosa*) is both an opportunistic plant and human pathogen. The plant pathogenic species *P. syringae* is highly diverse encompassing strains which can be differentiated from each other by symptomology or host range. These differences in pathogenicity are accounted for in the "pathovar" system of nomenclature adopted for use by plant pathologists (6). For instance, *P. syringae* pathovar (pv.) *glycinea* causes a disease of soybean while *P. syringae* pv. *tomato* causes a disease of tomato.

The first report on the composition of an EPS from a group I pseudomonad (*P. fluorescens*) appeared in 1956 (7). This EPS was reported to be a mannan. More recent studies indicated that laboratory varients of *P. fluorescens*, *P. putida* and *P. mendocina* are capable of producing alginate as an EPS (8). Alginate was reported previously to occur as an EPS of human pathogenic *P. aeruginosa* (9). Alginate is also a major structural polysaccharide of brown seaweed, which is the source of alginate used for all commercial applications. Alginate salts (called algin) or propylene glycol derivatives of alginate are used in the food, textile, paper and pharmaceutical industries among others. These uses are based on the water holding, gelling, emulsifying and stabilizing properties of alginates (3).

Structurally, alginates are a group of related polysaccharides composed of a linear backbone of 1,4-linked ß-D-mannuronic acid and its C5-epimer α-L-guluronic acid (10). The monomers can be present in varying ratios and as homopolymeric blocks (e. g., polymannuronate or polyguluronate) or heteropolymeric block structures. The relative amounts of the different block structures greatly affects the physical properties of the polymer. For instance, the presence of homopolymeric blocks of guluronate allows for the formation of brittle gels in the presence of calcium ions. Alginates isolated from pseudomonads differ from algal alginates in that they contain acetate groups and are thought to be devoid of homopolymeric blocks of guluronate (11).

Most studies on bacterial alginate synthesis and regulation have centered on *P. aeruginosa* due to the fatal consequences of the chronic lung infections of cystic fibrosis patients which are caused by this bacterium. Biochemical and molecular genetic techniques have been used to elucidate the biosynthetic pathway from fructose-6-phosphate to GDP-mannuronate and the genes responsible for encoding the responsible enzymes involved have been cloned (for a review, see 12). The subsequent polymerization, acetylation, export and epimerization steps are still not well

characterized. The control of alginate production in *P. aeruginosa* is highly complex with a large number of positive and negative regulatory genes being reported. One positive regulatory gene, *alg*R1, is a member of a class of environmentally responsive two-component regulatory genes and has an effector function. The *alg*R1 gene product activates transcription of the *alg*D gene (13).

Below we summarize our studies, which utilized over 200 bacterial strains, as well as the results of others on the nature, biosynthesis and regulation of EPS's produced by plant-associated group I pseudomonads.

NATURE OF THE EXOPOLYSACCHARIDES PRODUCED BY GROUP I PSEUDOMONADS

Alginates

Studies in our laboratory (14,15,16,17) as well as those of Gross and Rudolph (18,19,20) demonstrated that alginate is produced as an exopolysaccharide by several *Pseudomonas* species in addition to human pathogenic *P. aeruginosa* (Table 1). The bacterium *P. aeruginosa* isolated from a plant source as well as naturally-occurring strains of *P. fluorescens* are capable of alginate production.

Alginate synthesis can first be detected in early to mid-log phase dependent on the carbon source (20) and alginate is present in the form of a loosely-held slime layer and not as a tightly-held capsule. Initial yields of purified alginates were low (less than 1 g/L) (16, 17, 20), but much higher yields can be achieved using the proper strains and growth conditions (21, Fett and Wijey, unpublished). Several sugars and organic acids support alginate production *in vitro* by *P. syringae* pv. *phaseolicola* with gluconate and glycerol giving the highest yields (18). Sucrose was found to support alginate production by some strains, but the use of this carbon source can lead to production of levan, a neutral EPS, in addition to alginate (see below).

Alginates produced either *in vitro* or *in planta* are usually low in guluronic acid content (guluronate to mannuronate ratios less than 0.3) as determined by GC analyses (14,15,16,17). In contrast to human pathogenic *P. aeruginosa* where the alginates produced have a constant ratio of mannuronate to guluronate independent of growth conditions (11), the guluronic acid content of alginates produced by plant-associated pseudomonads is variable (16,17). Initial results using ^{1}H-NMR analyses indicated that alginates produced by *P. syringae* pv. *glycinea* may contain low levels of guluronate block structures (17), but these findings require confirmation. As stated above, alginates produced by human pathogenic *P. aeruginosa* are devoid of polyguluronic blocks and this was also demonstrated for alginates from single strains of *P. fluorescens*, *P. mendocina* and *P. putida* (11). The availability of pseudomonad alginates with blocks

Table 1
Alginate-Producing Plant-Associated Pseudomonads

P. aeruginosa[a]
P. cichorii
P. fluorescens[b]
P. syringae pathovars (*aptata, glycinea, lachrymans, morsprunorum, papulans, phaseolicola, pisi, savastanoi, syringae, tabaci, tagetis, tomato*)
P. viridiflava

[a] Isolated from a diseased onion bulb.

[b] Naturally-occurring strains isolated from plant rhizospheres.

Table 2
Effect of sodium chloride on alginate production

	μg alginate/mg dry cell wt (mg of alginate/liter)							
	Molar concentration of NaCl in the medium							
Bacterium	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
P. syringae pv. *glycinea* A-29-2m	13[a] (63)	16 (86)	35 (185)	52 (257)	85 (394)	117 (478)	100 (284)	79 (88)
P. viridiflava 671m	3 (16)	6 (36)	8 (61)	12 (63)	8 (38)	7 (31)	NT[b]	NT
P.fluorescens R4a-80	4 (22)	12 (73)	22 (122)	58 (139)	81 (121)	71 (105)	NT	NT

[a] Bacteria were cultured in modified King's medium B broth for 24 to 30 h at 28° C or 35° C (*P. fluorescens* only) with shaking (200 rpm).
[b] NT, not tested.

of guluronic acid would be desirable for some commercial applications which depend on the formation of hard, brittle gels.

The alginates produced by plant-associated pseudomonads are acetylated with values ranging from 1-18% as determined by colorimetric assay (14, 15,16,17, 19). Acetylation of alginates produced by human pathogenic *P. aeruginosa* is known to occur exclusively on the mannuronate residues with both mono- and di-acetylated forms (22).

The molecular mass of alginates produced *in vitro* and *in planta* by plant pathogenic group I pseudomonads ranges from 3 to 47 x 10^3 as determined by gel permeation-high-performance liquid chromatography (16,17). These values are low when compared to previously reported molecular masses of alginates produced by *P. aeruginosa* (23). Plant-associated group I pseudomonads are capable of producing the alginate-degrading enzyme alginate lyase (24, Fett, unpublished), and the action of this enzyme may be responsible for the low molecular masses of our preparations.

Also included in our studies were plant pathogenic pseudomonads of other rRNA-DNA homology groups. None of these bacteria were found to be capable of alginate production under the growth conditions tested (16).

Novel Acidic Exopolysaccharides

Based upon our initial results we had assumed that alginate was the acidic EPS produced by all group I pseudomonads. However, a recent study in our laboratory indicated that some strains produce novel acidic EPS's rather than alginate under the growth conditions tested (15). These additional EPS's have now been purified and their structures determined (Figures 1-3). The first to be characterized was given the trivial name of marginalan because the EPS was originally isolated from a strain of *P. marginalis*. Marginalan is a 1,3-linked galactoglucan substituted with pyruvate and succinate (Fig. 1) (25). The exact position on the glucose moiety substituted with succinate has not yet been determined. Marginalan is produced by several strains of *P. marginalis* and *P. fluorescens* (15) and EPS's with very similar compositions were reported for freshwater isolates of *P. fluorescens* and *P. putida* (26). EPS's with very similar structures have been reported for *Rhizobium meliloti* (27) and *Achromobacter* species (28). We have isolated an intracellular marginalan depolymerase from a

marginalan-producing strain of *P. marginalis* that acts by glucosidic cleavage (Osman et al., unpublished). The product of the depolymerase is a polysaccharide of approximately 2.5 x 10^5 D. The enzyme has a molecular mass of 28 kD and a pH optimum of 6.5. Two additional novel acidic polysaccharides are synthesized by certain strains of *P. marginalis* (Figs. 2 and 3) (29,30). As for marginalan, the acidic nature of these two EPS's is not due to the presence of uronic acids, but rather to the presence of acidic substituents.

Levan

Levan is a 2,6-linked ß-D-fructan with varying degrees of branching through O-1 (2). Several bacterial genera elaborate levan as an EPS when sucrose is the primary carbon source. This polysaccharide is synthesized exocellularly by the action of levansucrase on sucrose (2). Even though the ability to form domed, mucoid colonies on agar media containing high amounts of sucrose is considered diagnostic for the ability to form levan and is used as a taxonomic criterion to differentiate *Pseudomonas* species (5), actual chemical evidence for levan production by group I pseudomonads was very limited.

Recent work in our laboratory and in Rudolph's laboratory definitively showed that levan is produced as an EPS by several *Pseudomonas syringae* pathovars as well as by certain strains of *P. fluorescens* and *P. marginalis* when the growth medium contained sucrose (15,16,17,20). In sucrose-containing liquid media strains produce levan, levan and alginate, or alginate alone. Alginate production followed levan production for *P. syringae* pv. *phaseolicola* (20). Some group I pseudomonads (e.g., *P. aeruginosa, P. putida, P. viridiflava, P. cichorii*) are unable to produce levan (5)

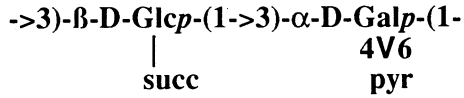

Figure 1. Structure of marginalan produced by *Pseudomonas marginalis* strain HT041B EPS.

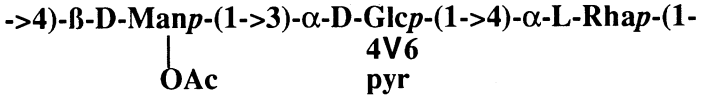

Figure 2. Structure of the acidic EPS produced by *P. marginalis* PF-05-2 and PM-LB-1.

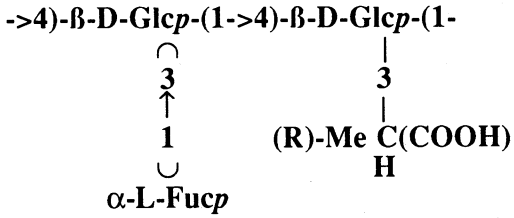

Figure 3. Structure of the acidic EPS produced by *P. marginalis* ATCC 10844.

including all *P. fluorescens* and *P. marginalis* strains which produce the acidic EPS marginalan (15).

EXOPOLYSACCHARIDE BIOSYNTHESIS

The biosynthetic pathway from fructose-6-phosphate to GDP-mannuronate utilized by human pathogenic *P. aeruginosa* and the corresponding biosynthetic genes (12) are shown in Figure 4.

In order to determine if the same pathway is functional in other group I pseudomonads, DNA probes were prepared using alginate genes cloned from human pathogenic *P. aeruginosa*. These probes were then used to screen for the presence of homologous sequences in chromosomal DNA preparations. Chromosomal DNA of the alginate-producing strains of *P. fluorescens*, *P. putida*, *P. cichorii*, *P. mendocina* and various *P. syringae* pathovars was shown to contain sequences with homology to the genes *alg*A, *alg*C and *alg*D (31, 32). Unexpectedly, strains of *P. fluorescens* and *P. marginalis* which produce acidic EPS's other than alginate also contained DNA with homology to the probes (31). This may indicate that these silent alginate genes are either defective or under regulatory controls which differ from the group I pseudomonads already demonstrated to produce alginate. A strain of *P. corrugata* (a non-fluorescent group I pseudomonad) was also shown to contain sequences with homology to the alginate genes (32). This species has not been demonstrated to produce alginate or any other EPS.

Additional support for this biosynthetic pathway being functional in group I pseudomonads other than human pathogenic *P. aeruginosa*, comes from the recent demonstration of phosphomannose isomerase activity in cell free extracts from *P. syringae* pv. *phaseolicola* (33). The mechanism for incorporation of guluronic acid into the polymer is not known. As for human pathogenic *P. aeruginosa*, other alginate-producing group I pseudomonads appear devoid of extracellular C-5 epimerase activity (24). For the pseudomonads, a membrane-bound epimerase may be involved or an epimerase may function at the nucleotide sugar level.

The biosynthesis of levan is unusual in that the responsible enzyme, levansucrase, acts extracellularly on the substrate sucrose (2). The presence of both intracellular and extracellular levansucrase was demonstrated for *P. syringae* pv. *phaseolicola* (20). Synthesis was constitutive and the enzyme had a pH optimum of 5.5 and a temperature optimum of 47° C (34). No information is available on the biosynthetic pathways leading to the three novel acidic EPS's produced by the group I pseudomonads.

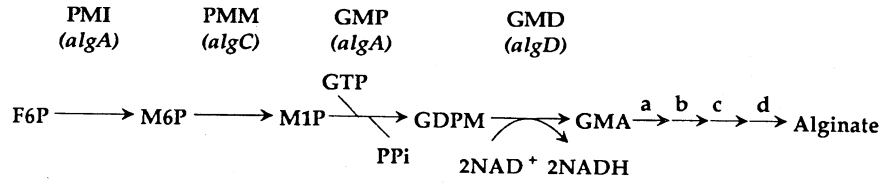

Figure 4. Alginate biosynthetic pathway in *P. aeruginosa* (12). Abbreviations: F6P, fructose-6-phosphate; M6P, mannose-6-phosphate, M1P, mannose-1-phosphate; GDPM, GDP-mannose; GMA, GDP-mannuronic acid; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-mannose pyrophos-phorylase; GMD, GDP-mannose dehydrogenase. Gene *alg*A encodes a bifunctional enzyme. The polymerization (a), acetylation (b), export (c) and epimerization (d) steps are undefined.

Table 3
Effect of ionic (NaCl) and nonionic (sorbitol) solutes on alginate production by *Pseudomonas syringae* pv. *glycinea*

Solute	Strain	
	A-29-2m	NCPPB 2159
Control		
µg/mg dry cell wt	32[a]	98
mg/L	194	602
Sorbitol (0.8 M)		
µg/mg dry cell wt	171	287
mg/L	1116	1616
NaCl (0.4 M)		
µg/mg dry cell wt	179	269
mg/L	1256	1176

[a] Bacteria were cultured in modified King's medium B broth for 24 to 30 h at 28° C with shaking (200 rpm).

ENVIRONMENTAL FACTORS AFFECTING EPS PRODUCTION

As found for human pathogenic *P. aeruginosa* (35), optimal temperatures for alginate production by the plant-associated group I pseudomonads are often lower than those optimal for growth (15).

Increased osmolarity has been reported to increase *algD* promoter activity possibly mediated by the positive regulatory gene *algR1* (12). As DNA sequences with homology to *algR1* were detected in other alginate-producing group I pseudomonads (31,32) we examined the effect of sodium chloride on alginate production by two plant pathogenic pseudomonads (*P. syringae* pv. *glycinea* and *P. viridiflava*) as well as one saprophytic pseudomonad (*P. fluorescens*). Sodium chloride was found to stimulate alginate production by the three strains (Table 2).

To distinguish between an ionic versus an osmotic effect due to the addition of sodium chloride to the medium, the nonionic, nonmetabolizable solute sorbitol was also tested for its ability to increase alginate production by two strains of *P. syringae* pv. *glycinea*. A similar stimulation of alginate production occurred with sorbitol for both strains indicating that the increase in alginate production was due to an increase in osmolarity (Table 3). The role of the regulatory gene *algR1* in the response of these bacteria to increased osmolarity is not known.

Dehydration due to addition of ethanol to the medium has also been reported to transcriptionally activate *algD* and to increase alginate production by *P. aeruginosa* (36). We determined that addition of ethanol to the medium also increased alginate production by *P. syringae* pv. *glycinea*, *P. viridiflava* and *P. fluorescens* (Table 4). This stimulatory effect may be due to membrane disruption.

DISCUSSION

It is now clear that several acidic EPS's are produced by rRNA group I pseudomonads. Alginates are the most common acidic EPS's, but novel acidic EPS's are also synthesized by certain strains of *P. fluorescens* and *P. marginalis*. These two bacteria are very closely related, differing primarily in pectolytic activity. Examination of additional strains of these species may lead to the identification of additional novel acidic EPS's. No strain produced more than one type of acidic EPS, although the novel

Table 4
Effect of ethanol on alginate production

Bacterium	μg alginate/mg dry cell wt (mg alginate/liter) Concentration of ethanol in medium (%)					
	0	1	2	3	4	5
P. syringae pv. *glycinea* A-29-2m	20[a] (118)	53 (264)	81 (349)	117 (434)	83 (155)	52 (44)
P. viridiflava 671m	5 (30)	44 (231)	69 (354)	76 (338)	32 (69)	18 (18)
P. fluorescens R4a-80	9 (53)	25 (89)	40 (43)	52 (38)	48 (38)	42 (34)

[a] Bacteria were cultured in modified King's medium B broth for 24 to 30 h at 28 or 35° C (*P. fluorescens* only) with shaking (200 rpm).

acidic EPS-producing bacteria appear to contain the structural genes required for alginate biosynthesis. The ability to produce more than one acidic EPS for an alginate-producing bacterium has been demonstrated for *Azotobacter chroococcum* (37) and *A. beijerinckii* (38). The additional acidic EPS produced by *A. chroococcum* has a similar composition to the acidic EPS produced by *P. marginalis* strains PF-05-2 and PM-LB-1. The only neutral EPS found to be synthesized by the group I pseudomonads was levan. No strains of *P. fluorescens* were found to produce a mannan as an EPS as reported by Eagon (7). Members of the other *Pseudomonas* rRNA groups do not appear to be capable of producing alginate.

The biosynthetic pathway from fructose-6-phosphate to GDP-mannuronic acid appears to be common to the group I pseudomonads based primarily on DNA sequence homologies. To date only low levels of one of the three enzymes involved (one protein being bifunctional) has been shown to occur in a plant-associated group I pseudomonad. Very low levels of these enzymes also occur in human pathogenic *P. aeruginosa* and their isolation and characterization required the cloning of the respective genes and their overexpression in either *P. aeruginosa* or *Escherichia coli* (12). A similar strategy may be required for the other group I pseudomonads.

Currently, of the EPS's produced by the group I pseudomonads, alginates appear to have the greatest possibility for commercialization. These alginates may replace algal alginates at least for some uses such as viscosifying and stabilizing agents and may be used as therapeutic agents. For instance, alginates high in mannuronate were recently shown to stimulate production of cytokines by human monocytes (39) and to have antitumor activity (41). In order for the commercialization of *Pseudomonas* alginates to become a reality, higher yields of alginates from industrially-acceptable carbon sources such as glucose and fructose must be obtained. The identification of strains which produce alginates containing homopolymeric blocks of guluronate would hasten commercialization of pseudomonad alginates where the ability to form hard, brittle gels is required, although the recent cloning of the gene which encodes for the C-5 epimerase of an alginate-producing strain of *A. vinelandii* may allow for the tailoring of alginates for specific uses (39). Many industrial uses for levans have also been proposed based on their low viscosity and high water solubility, but levans are not yet in commercial production (42).

The ever increasing knowledge of environmental factors which affect EPS production by group I pseudomonads and of the structural and regulatory genes

involved in EPS synthesis should greatly improve the chances for future commercialization.

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Chapter 7

THE BEHAVIOR OF STARCH BIOPOLYMERS DERIVED FROM DIFFERENT GENETIC VARIETIES OF MAIZE

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ABSTRACT

Starch biopolymers comprise the principle energy storage polymer system in plants. Throughout history they have been utilized as a source of energy by humans, as well. As society has become more complex, however, the value of starches as functional food and industrial ingredients has increased. Recent advances in polymer science have permitted the identification of some structural characteristics which influence starch biopolymer functionality. Progress in biochemistry has also revealed some of the genetically controlled biosynthetic mechanisms which determine the formation of functionally important structural characteristics. Consequently, it is now becoming possible to select behavior qualities of some starch biopolymers based on the genetic variety of their plant source. This paper presents some examples of starch biopolymer behavioral and structural characteristics. The strategic employment of such native starch biopolymers permits greater flexibility in the use of modified starches.

INTRODUCTION

Starch is the major energy storage polymer in the plant kingdom. Starch may be characterized as a family of biopolymers whose repeating unit, or monomer, is *alpha*-D-glucose. Glucose is polymerized through acetal linkages with adjacent glucose

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